Note

12-*O*-Tetradecanoylphorbol 13-acetate promotes proliferation and epithelial–mesenchymal transition in HHUA cells cultured on collagen type I gel: A feasible model to find new therapies for endometrial diseases

Yusuke Hanaki,[1*] Sena Miyoshi,[2] Yasunori Sugiyama,[1] Ryo C. Yanagita,[1] and Masashi Sato[1]

[1]Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Kagawa 761-0795, Japan. [2]Division of Applied Biological and Rare Sugar Sciences, Graduate School of Agriculture, Kagawa University, Kagawa 761-0795, Japan.

* Correspondence: Yusuke Hanaki, hanaki.yusuke@kagawa-u.ac.jp

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Running head: Properties of HHUA cells cultured on collagen type I gel

Abstract

HHUA endometrial adenocarcinoma cells aggregated into spheroids when cultured on collagen type I gels. 12-*O*-Tetradecanoylphorbol 13-acetate, a PKC activator, disassembled the spheroids through epithelial–mesenchymal transition and increased their proliferation rate, while inducing cell death under monolayer culture conditions. These unusual behaviors of endometrial epithelial cells with collagen fibers could be a target for the treatment of some endometrial diseases. (60 words)

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The endometrium is the inner lining of the uterus and has functional and basal layers. The functional layer is composed of epithelial and stromal cells which repeat the cycle of proliferation, differentiation into secretory glands and decidua, and shedding, in response to the secretion of estrogens and progesterone from the ovaries. The hyperplasia and malignant transformation of endometrial epithelial cells lead to endometrial cancer, one of the most common gynecological cancers (Ryan *et al*. 2005). The invasion of tumor cells into the myometrium causes initial symptoms such as metrorrhagia, micturition pain, and abdominal pain. These cells further metastasize to the cervix, lymph nodes, and other remote tissues in later stages. In addition, endometrial cells frequently invade the myometrium without malignant transformation. Adenomyosis is a common and benign gynecological disorder seen in approximately 20% of women of reproductive age (Naftalin *et al*. 2012). In the uterus of patients, endometrial cells exist in the myometrium and form ectopic endometrial glands. A recent study on the 3D morphology of adenomyotic tissue showed that endometrial glands directly invade the myometrium and form an ant colony-like network (Yamaguchi *et al*. 2021). Since the ectopic endometrium also repeats along with the menstrual cycle, patients suffer from annoying symptoms including menstrual pain and abnormal uterine bleeding.

Since the proliferation of endometrial cells is dependent on estrogens, endocrine therapies including progestin-estrogen formulations, gonadotropin-releasing hormone (GnRH) agonists/antagonists, and progestins are used for the treatment of adenomyosis with the intent to inhibit estrogen production (Vannuccini *et al*. 2018). These agents reduce symptoms but do not eliminate the lesion, and the symptoms recur after discontinuation of the medication. Thus, the long treatment period is burdensome for patients. Moreover, hormone therapies suppress ovulation and menstruation, resulting in impeded fertility during the dosing period. Therefore, there is no effective therapy for patients seeking pregnancy. On the other hand, hysterectomy is the main treatment for endometrial cancer. The prognosis is relatively good because the clear subjective symptoms allow for early detection. However, endocrine therapies are also applied to early-stage patients seeking pregnancy to preserve their fertility, though the high recurrence rate remains a problem (Gunderson *et al*. 2012). Considering these dissatisfactions with current therapies, it is desired to develop a novel treatment without inhibiting hormone secretion.

Since the invasion of endometrial cells into the myometrium is a pathological feature of adenomyosis and endometrial cancer, we considered that the microenvironment of the invasion front can be a potential treatment target for these diseases. While endometrial epithelial cells are normally

aligned on the basement membrane, tumorigenic cells acquired weakened cell-cell adherence, reduced polarity, and an enhanced migration ability via epithelial–mesenchymal transition (EMT) (Abal *et al*. 2007). High expression levels of matrix metalloproteinases (MMPs) and discontinuation of the basement membrane were also observed on the invasive front of endometrial cancer (Planagumà *et al*. 2011). On the other hand, the process of benign invasion of the endometrium in adenomyosis remains more controversial because both epithelial and stromal cells invade the myometrium and retain normal glandular structure. However, EMT was also reported to be involved in the progression of adenomyosis (Chen *et al*. 2010). Moreover, some research demonstrated that patients with adenomyosis showed higher levels of MMPs in the uterine cavity (Inagaki *et al*. 2003), and the loss of lamin and collagen type IV, which are main components of the basement membrane, was observed in adenomyosis lesions (Shiina *et al*. 2002). These results suggest that adenomyosis has some similar characteristics to malignant diseases.

Based on the fact that the basement membrane was disrupted in the lesions of endometrial cancer and adenomyosis, we assumed that the invasive epithelial cells are in contact with collagen type I (COL-I), a major component of the extracellular matrix of the stroma and myometrium (Wolańska *et al*. 1998), and that the COL-I-rich microenvironment may contribute to the progression of these diseases. Recent studies revealed that fibrosis is significantly increased in adenomyotic lesions (Liu *et al*. 2016; Kobayashi *et al*. 2020). Periodic inflammation associated with menstruation is estimated to induce fibroblast to myofibroblast transition (FMT) in adenomyotic stroma and myometrium, resulting in overproduction of COL-I. It was also reported that COL-I enhanced migration of stromal cells in a spheroid model of endometriosis (Stejskalová *et al*. 2021), but the effects of COL-I on endometrial epithelial cells and its relation to progression of endometrial disorders remain unclear. To validate our hypothesis that the contact between COL-I and endometrial epithelial cells would promote these diseases, we in this study explored characteristic features of endometrial epithelial cells in contact with COL-I. We here report that COL-I would protect endometrial epithelial cells from inflammation-induced cell death, and rather support their proliferation following EMT. These findings will serve as novel targets of drug screening for endometrial diseases.

In this study, we cultured HHUA endometrial cells on COL-I, and evaluated whether the contact with COL-I affects the properties of endometrial epithelial cells. The HHUA cell line was established from a well-differentiated endometrial adenocarcinoma (Ishiwata *et al*. 1984). These cells were reported to maintain estrogen and progesterone receptors despite cancer cells, and thus seemed to be

a good model for studying both benign and malignant endometrial diseases. The HHUA cell line (RCB0658) was provided by RIKEN BRC through the National Bio-Resource Project of MEXT/AMED (Ibaraki, Japan), and cultured in Ham's F-12 medium (Merck, Darmstadt, Germany) with 10% heat-inactivated FBS (Corning, NY, USA, or BioWest, Maine-et-Loire, France), 1 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) in a 5% CO_2 humidified incubator maintained at 37°C.

Firstly, we seeded HHUA cells ($2.5x10^4$ cells/well) in non-coated and COL-I gel-coated 24-well plates, and after 48 h observed their morphology using phase-contrast microscopy (Figure 1A, B). To prepare the COL-I gel-coated plate, 0.2% atelocollagen solution (3D-LG, lot No. 362011, KOKEN Co., Ltd, Tokyo, Japan) was added to each well of a non-coated culture plate (150 μL/cm^2) and incubated at 37°C for 2 h to allow gelation. As shown in Figure 1A, HHUA cells were polygonal in shape on the non-coated culture plate. On the other hand, culture on the COL-I gel induced spheroid formation (Figure 1B). The cell aggregation was confirmed by the nucleic acid stain using DAPI (Figure S1). Compared to the culture condition using a non-coated plate, the cell proliferation rate increased on a COL-I gel during long-term culture (24–48 h) rather than initial stage of culture (0–24 h) (Figure S2). Therefore, the spheroid could escape from contact inhibition and growth in a high cell density condition. Interestingly, HHUA cells cultured on a commercial COL-I-coated plate formed a monolayer with a polygonal cell shape similar to those on a non-coated plate (Figure S3). The COL-I coating gives only a thin layer without a 3D fibril network because it is prepared from low-density COL-I (< 10 μg/cm^2 bottom surface). On the other hand, our COL-I gel-coated plate was prepared from 300 μg of COL-I per cm^2 of bottom surface and forms 3D fibril networks. The minimum concentration of atelocollagen required to form a gel was about 0.1%, and we used a 0.2% solution to obtain a more stable gel that would not be stripped during cell seeding and medium exchange. Therefore, the 3D structure of COL-I fibers would be important for spheroid formation of HHUA cells.

Considering that cell spheroids are normally generated by suspension culture using a non-adhesion plate, we estimated that a COL-I gel is unsuitable as a scaffold for endometrial epithelial cells, resulting in self-aggregation. Ohbayashi *et al*. previously reported that MCF-7 breast adenocarcinoma cells formed spheroids when they were cultured on a COL-I coated plate, while Caco-2 (colorectal adenocarcinoma) and NCI-H292 (lung mucoepidermoid carcinoma) cells did not (Ohbayashi *et al*. 2008). It is interesting that the spheroid formation ability was found only in MCF-7, which is derived from estrogen- and progesterone-receptor positive adenocarcinoma similar to HHUA. To our

knowledge, there are few reported examples of cell lines that form spheroids just by being seeded on COL-I. We continue to investigate the mechanism underlying the COL-I gel-induced alteration in the morphology of HHUA cells.

Next, we examined the effects of some drugs on the properties of HHUA cells cultured on a COL-I gel. We first treated HHUA cells with 17β-estradiol that is related to the progression of various gynecological diseases, but any obvious responses were not observed. The endometrial cells in the uterus are periodically stimulated by some cytokines since the endometrium is exposed to menstrual bleeding (Kyama *et al*. 2006). We estimated that proinflammatory cytokines, or both cytokines and estrogen, are necessary for progression of endometrial disorders. We next used 12-*O*-tetradecanoylphorbol 13-acetate (TPA), an inflammatory agent that activates protein kinase C (PKC) and causes inflammation by inducing the production of proinflammatory cytokines (Kontny *et al*. 1999). It was also reported that 17β-estradiol increased PKC activity in endometrial cancer cells (Fujimoto *et al*. 1996). Therefore, we considered that PKC activation by TPA could mimic inflammatory and hormonal stimuli that ordinarily occur in the endometrium.

On the non-coated plate, cell debris was observed 24 h after TPA (10 nM) treatment, though some viable cells simultaneously showed a morphological change (Figure 1C). The effects of TPA on the proliferation of HHUA cells were examined by the WST-8-based cytotoxicity assay. HHUA cells (5,000 cells/well) were seeded in 96-well plate and allowed to attach for 24 h. Thereafter, they were treated with TPA (1, 10, 100 nM) for an additional 48 h, and the cell number was estimated with Cell Counting Kit-8 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The cytotoxicity assay also suggested that TPA significantly induced cell death in HHUA cells cultured on non-coated or commercial COL-I coated plates (Figure 2, S3). TPA showed a biphasic dose-response curve with maximal cytotoxicity at 10 nM as reported previously in other cell lines (Keck *et al*. 2012). This effect could be attributed to the fact that TPA rapidly downregulates PKC at high concentrations (Adams and Gullick 1989). On the other hand, TPA disassembled the spheroid of HHUA cells on COL-1 gel. The TPA-treated cells were scattered and showed a fibroblast-like spindle shape (Figure 1D). The cell process extension was also observed probably due to the reorganization of microtubules as reported in estrogen receptor-positive MCF-7 cells (Platet *et al*. 1998). These effects of TPA were canceled under coexistence with bisindolylmaleimide I (Bis-I, 1 μM), a PKC inhibitor, indicating that the effect was not due to non-specific toxicity but to PKC signaling (Figure 1F). Moreover, TPA significantly enhanced the proliferation of HHUA cells on COL-I gels as opposed to the case of the monolayer

culture (Figure 2). The proliferation rate was maximal at lower concentration (1 nM). Because collagens were suggested to activate PKC via collagen receptors such as integrins and discoidin domain receptors (DDRs) and DDR2 was upregulated by PKC (Vonk *et al*. 2011; Yen *et al*. 2014), PKC might be more potently activated and downregulated in cells cultured on a COL-I gel.

As mentioned above, EMT is a key process of invasion of endometrial cancer and adenomyosis. In addition, TPA was reported to induce EMT in some cancer cell lines (He *et al*. 2010; Kamiya *et al*. 2016). Thus, we examined the effects of TPA on the expression of EMT marker proteins in HHUA cells cultured on COL-I gel. HHUA cells ($7.5x10^4$ cells/well) were seeded in a COL-I gel-coated 48-well plate. After preincubation for 20 h, they were treated with TPA (10 nM) for another 24 h. They were lysed by 2X SDS buffer and subjected to SDS-PAGE followed by western blotting. As shown in Figure 3, TPA reduced the expression of E-cadherin, a key protein maintaining the cell-cell junction of epithelial cells, by approximately 50%. On the other hand, vimentin, an intermediate filament protein of stromal cells, was increased by about 50%, though the increment was not statistically significant ($p = 0.09$). Consequently, the ratio of vimentin to E-cadherin was significantly increased by TPA treatment, suggesting that the disassembly of spheroids and fibroblast-like morphology of TPA-treated cells would be attributed to EMT. It was reported that reactive oxygen species and histone acetylation were involved in TPA-induced EMT in MCF-7 cells (Kamiya *et al*. 2016). Moreover, in PC-3 and A549 cells, GSK-3β inhibition by PKC promoted the stabilization and transcription of Snail, a transcription factor that decreases E-cadherin and increases vimentin (Liu *et al*. 2014). The PKC/ERK pathway is also suggested to mediate thrombin-induced EMT in A549 cells (Song *et al*. 2013). Therefore, these signal transduction molecules may serve as new therapeutic targets for treatment of endometrial diseases.

In this study, we revealed that PKC activation promoted proliferation and EMT in HHUA cells cultured on a COL-I gel, but induced cell death when they were cultured under monolayer culture conditions. COL-I is a major component of stromal fibrils and thus ordinarily plays a role as a scaffold of fibroblasts. On the contrary, endometrial epithelial cells maintain contact with one another and construct the monolayer on the basement membrane. We predicted that TPA-treated HHUA cells lost their epithelial morphology and acquired a fibroblast-like character through EMT, and thus they favorably proliferated on a COL-I gel but not under monolayer culture conditions. These results can reflect some aspects of the progression of adenomyosis and endometrial cancer. Namely, the inflammatory stimuli by menstruation should normally induce cell death and shedding of endometrial

epithelial cells, but oppositely increase their proliferation and migration when they contact COL-I fibers. The unusual behavior of endometrial epithelial cells on COL-I gel can be a new treatment target for endometrial cancer and adenomyosis, since the contact between endometrial epithelial cells and COL-I fibers would be a specific event in the invasive front of these lesions.

It seems to be difficult to make *in vivo* experimental models of endometrial diseases that exhibit periodic inflammation accompanied by fibrogenesis, because menstruation is found only in humans and a limited number primates such as apes, old world monkeys, and new world monkeys (Critchley *et al*. 2020). Among rodents, only the spiney mouse *Acomys cahirinus* has been reported to have menstruation (Bellofiore *et al*. 2017). Therefore, our *in vitro* model will help determine the mechanism underlying the inflammation-induced EMT and the role of COL-I in the progression of endometrial disorders. We have also initiated drug screening to identify compounds that inhibit TPA-induced EMT or show specific cytotoxicity to HHUA cells cultured on a COL-I gel. EMT is easily detected by the obvious morphological change from spheroids to scattered spindle cells, and the WST-8-based cytotoxicity assay is easily applicable to cells cultured on a COL-I gel-coated plate. These studies will contribute to developing a new approach to treat endometrial diseases without inhibition of hormone secretion.

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Supplementary material

Supplementary material is available at Bioscience, Biotechnology, and Biochemistry online. Figure S1, S2 and S3 are included in the Supplementary material.

Data availability

The data underlying this article are available in the article and its online supplementary material.

Author contributions

Y.H. conceived and designed the research. Y.H., S.M., and Y.S. performed the experiments and analyzed the data. All authors interpreted the results of the experiments. Y.H. wrote the original draft.

R.C.Y., Y.S., and M.S. revised the manuscript.

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Disclosure statement

The authors declare no conflicts of interest.

References

Abal M, Llauradó M, Doll A et al. Molecular determinants of invasion in endometrial cancer. *Clin Trans Oncol* 2007;9:272–7.

Adams JC and Gullick WJ. Differences in phorbol-ester-induced down-regulation of protein kinase C between cell lines. *Biochem J* 1989; 257:905–11.

Bellofiore N, Ellery SJ, Mamrot J et al. First evidence of a menstruating rodent: the spiny mouse (Acomys cahirinus). *Am J Obstet Gynecol* 2017;216:40.e1-40.e11.

Chen Y-J, Li H-Y, Huang C-H et al. Oestrogen-induced epithelial-mesenchymal transition of endometrial epithelial cells contributes to the development of adenomyosis. *J Pathol* 2010;222:261–70.

Critchley HOD, Babayev E, Bulun SE et al. Menstruation: science and society. *Am J Obstet Gynecol* 2020;223:624–64.

Fujimoto J, Hori M, Ichigo S et al. Estrogen induces expression of c-fos and c-jun via activation of protein kinase C in an endometrial cancer cell line and fibroblasts derived from human uterine endometrium. *Gynecol Endocrinol* 1996;10:109–18.

Gunderson CC, Fader AN, Carson KA et al. Oncologic and reproductive outcomes with progestin therapy in women with endometrial hyperplasia and grade 1 adenocarcinoma: a systematic review. *Gynecol Oncol* 2012;125:477–82.

He H, Davidson AJ, Wu D et al. Phorbol ester phorbol-12-myristate-13-acetate induces epithelial to mesenchymal transition in human prostate cancer ARCaPE cells. *The Prostate* 2010;70:1119–26.

Inagaki N, Ung L, Otani T et al. Uterine cavity matrix metalloproteinases and cytokines in patients with leiomyoma, adenomyosis or endometrial polyp. *Eur J Obstet Gynecol Reprod Biol*

2003;111:197–203.

Ishiwata I, Ishiwata C, Soma M et al. Establishment of human endometrial adenocarcinoma cell line containing estradiol-17 beta and progesterone receptors. *Gynecol Oncol* 1984;17:281–90.

Kamiya T, Goto A, Kurokawa E et al. Cross Talk Mechanism among EMT, ROS, and Histone Acetylation in Phorbol Ester-Treated Human Breast Cancer MCF-7 Cells. *Oxid Med Cell Longev* 2016;2016:1284372.

Keck GE, Poudel YB, Rudra A et al. Role of the C8 gem-dimethyl group of bryostatin 1 on its unique pattern of biological activity. *Bioorg Med Chem Lett* 2012;22:4084–88.

Kobayashi H, Kishi Y and Matsubara S. Mechanisms Underlying Adenomyosis-Related Fibrogenesis. *Gynecol Obstet Invest* 2020;85:1–12.

Kontny E, Łkowska MZ, Ryzewska A et al. Protein kinase C-dependent pathway is critical for the production for the production of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6). *Cytokine* 1999;11:839-48.

Kyama CM, Overbergh L, Debrock S et al. Increased peritoneal and endometrial gene expression of biologically relevant cytokines and growth factors during the menstrual phase in women with endometriosis. *Fertil Steril* 2006;85:1667–75.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.

Liu X, Shen M, Qi Q et al. Corroborating evidence for platelet-induced epithelial-mesenchymal transition and fibroblast-to-myofibroblast transdifferentiation in the development of adenomyosis. *Human Reprod* 2016;31:734–49.

Liu Z, Chen X, Song H et al. Snail regulated by PKC/GSK-3β pathway is crucial for EGF-induced epithelial-mesenchymal transition (EMT) of cancer cells. *Cell Tissue Res* 2014;358:491-502.

Naftalin J, Hoo W, Pateman K et al. How common is adenomyosis? A prospective study of prevalence using transvaginal ultrasound in a gynaecology clinic. *Hum Reprod* 2012;27:3432–9.

Ohbayashi M, Yasuda M, Kawakami I et al. Effect of interleukins response to ECM-induced acquisition of drug resistance in MCF-7 cells. *Exp Oncol* 2008;30:276–82.

Planagumà J, Liljeström M, Alameda F et al. Matrix metalloproteinase-2 and matrix metalloproteinase-9 codistribute with transcription factors RUNX1/AML1 and ETV5/ERM at the invasive front of endometrial and ovarian carcinoma. *Hum Pathol* 2011;42:57–67.

Platet N, Prévostel C, Derocq D et al. Breast cancer cell invasiveness: correlation with protein kinase C activity and differential regulation by phorbol ester in estrogen receptor-positive and -negative cells. *Int J Cancer* 1998;75:750-756.

Ryan AJ, Susil B, Jobling TW et al. Endometrial cancer. *Cell Tissue Res* 2005;322:53–61.

Shiina M, Obata K, Noda K et al. Neoplastic aspects of adenomyosis: an immunohistochemical study on the basement membrane and extracellular matrix proteinase. *Fertil Steril* 2002;77:S19.

Song JS, Kang CM, Park, CK et al. Thrombin induces epithelial-mesenchymal transition via PAR-1, PKC, and ERK1/2 pathways in A549 cells. *Exp Lung Res*. 2013;39:336-48.

Stejskalová A, Fincke V, Nowak M et al. Collagen I triggers directional migration, invasion and matrix remodeling of stroma cells in a 3D spheroid model of endometriosis. *Sci Rep* 2021;11:4115.

Sugiyama Y, Katayama S, Kameshita I et al. Expression and phosphorylation state analysis of intracellular protein kinases using Multi-PK antibody and Phos-tag SDS-PAGE. *MethodsX*. 2015;2:469–74.

Vannuccini S, Luisi S, Tosti C et al. Role of medical therapy in the management of uterine adenomyosis. *Fertil Steril* 2018;109:398–405.

Vonk LA, Doulabi BZ, Huang CL et al. Collagen-induced expression of collagenase-3 by primary chondrocyte is mediated by integrin α1 and discoidin domain receptor 2: a protein kinase C-dependent pathway. *Rheumatology* 2011;50:463-72.

Wolańska M, Sobolewski K, Drozdzewicz M et al. Extracellular matrix components in uterine leiomyoma and their alteration during the tumour growth. *Mol Cell Biochem* 1998;189:145–52.

Yamaguchi M, Yoshihara K, Suda K et al. Three-dimensional understanding of the morphological complexity of the human uterine endometrium. *iScience* 2021;24:102258.

Yen TL, Lu WJ, Lien LM et al. Amarogentin, a secoiridoid glycoside, abrogates platet activation through PLCγ2-PKC and MAPK pathways. *BioMed Res Int* 2014;2014:728019.

Figures

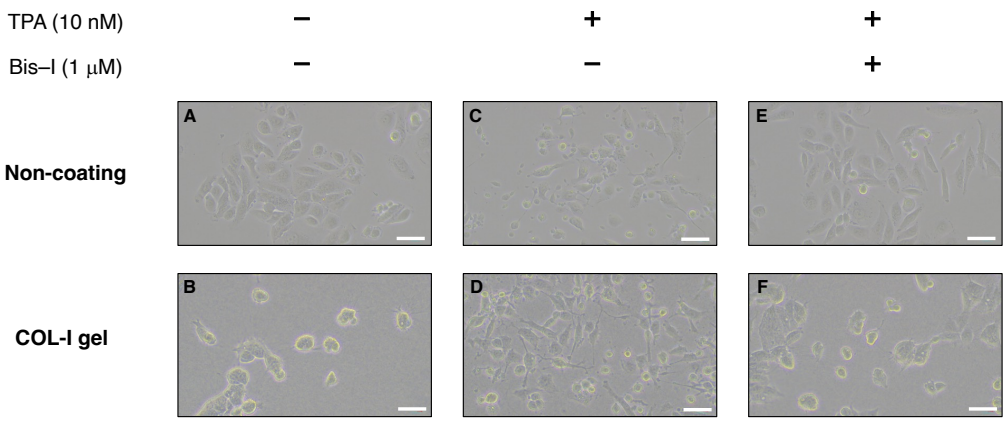

Figure 1. Effects of a COL-I gel and TPA on the morphology of HHUA cells.
HHUA cells ($2.5x10^4$ cells/well) were seeded in non-coated or COL-I gel-coated 24-well plates. After preincubation for 24 h, TPA solution in DMSO or DMSO alone was added and incubated for another 24 h. Bisindolylmaleimide I (Bis-I) solution in DMSO was added 30 min before being treated with TPA (final concentration of DMSO, 0.2%). These cells were observed using a phase-contrast microscope (ECLIPSE Ts2; NIKON, Tokyo, Japan). Scale bar: 50 μm.

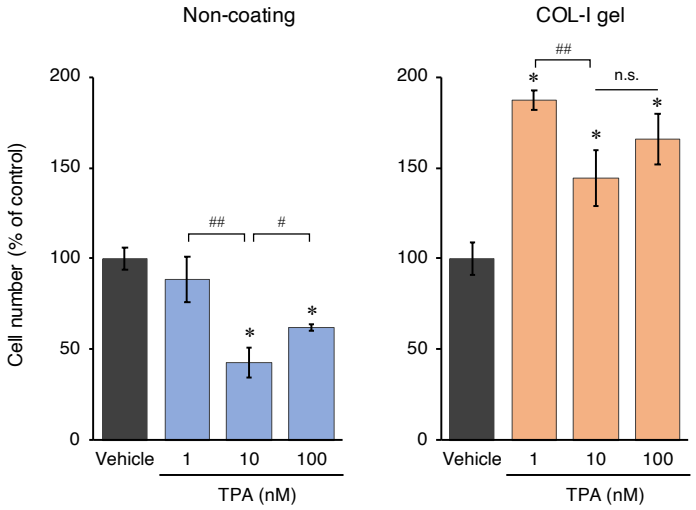

Figure 2. Effects of TPA on the proliferation of HHUA cells.
HHUA cells (5,000 cells/200 μL·well) were seeded in a 96-well plate. For an assay using a COL-I gel-coated plate, 50 μL of 0.2% atelocollagen solution was added to each well of a 96-well plate and incubated at 37°C for 2 h, before HHUA cells (5,000 cells/150 μL·well) were seeded. After preincubation for 24 h, a 1-μL solution of TPA in DMSO or DMSO alone was added. After incubation for 48 h, 20 μL of Cell Counting Kit-8 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added to each well, and the plate was incubated at 37°C for another 4 h. The absorbance was measured at 450 nm using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Walthman, MA, USA). The cell number in the presence of each concentration of TPA was plotted as a percentage relative to the vehicle group. Error bars represent standard deviation ($n = 4$). * $p < 0.001$ vs vehicle group, $^{\#\#}$ $p < 0.01$, $^{\#}$ $p < 0.05$, n.s., not significant (Tukey's test).

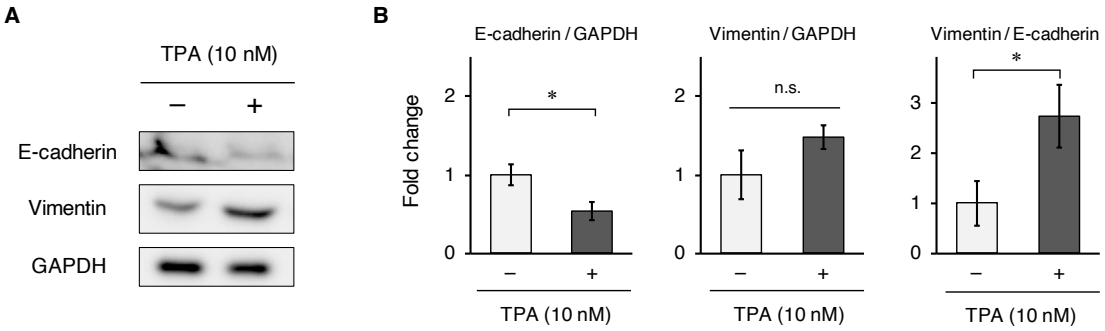

Figure 3. TPA induced EMT in HHUA cells cultured on a COL-I gel.

(A) A representative result of western blotting and (B) quantification of band intensity from triplicate samples are shown. HHUA cells ($7.5x10^4$ cells/well) were seeded in a COL-I gel-coated 48-well plate. After preincubation for 20 h, TPA solution in DMSO or DMSO alone was added (final concentration of DMSO, 0.1%), and incubated for another 24 h. Cells were washed with PBS, lysed by adding 100 μL of 2X SDS sample buffer, and boiled for 10 min. SDS-PAGE was performed according to the method of Laemmli (Laemmli 1970) in slab gels consisting of a 10% acrylamide separation gel and a 3% stacking gel. Western blotting was performed as described previously (Sugiyama *et al*. 2015). Anti-E-cadherin (24E10), anti-vimentin (D21H3), anti-GAPDH (D16H11), and HRP-conjugated anti-rabbit IgG antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). These antibodies were diluted 1:1000 and used for the detection of immunoreactive bands. The intensity of each band was quantified using Amersham Imager 680 analysis software (GE healthcare, Chicago, IL, USA). Error bars represent standard deviation ($n = 3$). n.s., not significant, * $p < 0.05$ (Welch's *t*-test).

Supplementary material

12-*O*-Tetradecanoylphorbol 13-acetate promotes proliferation and epithelial–mesenchymal transition in HHUA cells cultured on collagen type I gel: A feasible model to find new therapies for endometrial diseases

Yusuke Hanaki,[1*] Sena Miyoshi,[2] Yasunori Sugiyama,[1] Ryo C. Yanagita,[1] and Masashi Sato[1]

[1]Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Kagawa 761-0795, Japan. [2]Division of Applied Biological and Rare Sugar Sciences, Graduate School of Agriculture, Kagawa University, Kagawa 761-0795, Japan.

* Correspondence: Yusuke Hanaki, hanaki.yusuke@kagawa-u.ac.jp

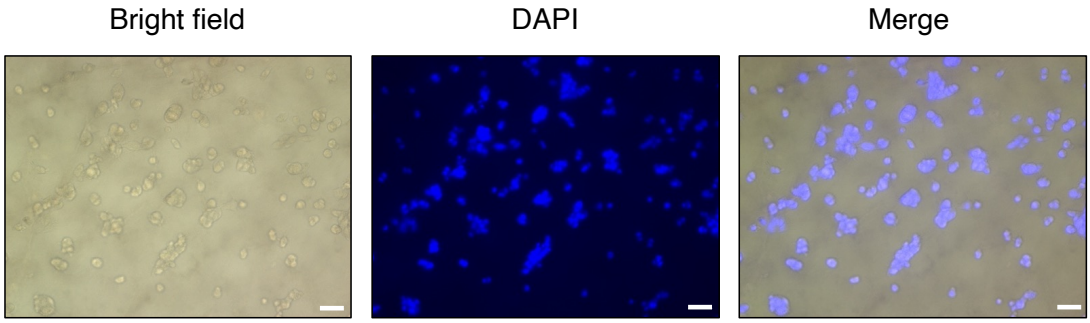

Figure S1. Aggregation of HHUA cells cultured on a COL-I gel.
HHUA cells were seeded at approximately 50% confluence in a COL-I gel coated glass bottom dish. After 24 h incubation, the cells were fixed with 4% paraformaldehyde in PBS for 20 min, then permeabilized with 0.1% triton-X in PBS for 5 min. The cells were washed with PBS, and incubated with 1mg/mL DAPI solution in PBS for 15 min, and observed using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). Scale bar: 50 μm.

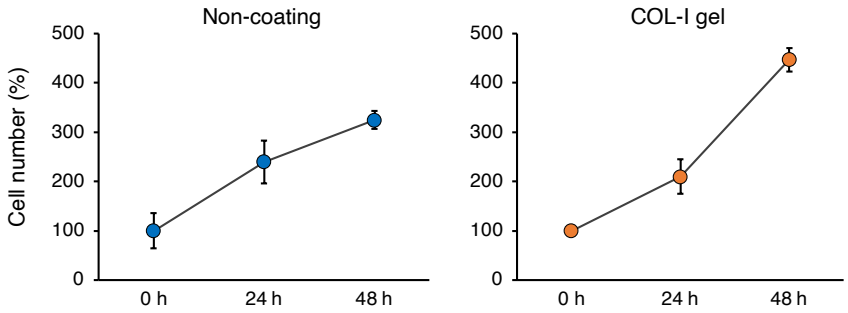

Figure S2. Proliferation of HHUA cells cultured on non-coated and COL-I gel-coated plates.
HHUA cells (5,000 cells/200 μL·well) were seeded in a 96-well plate and allowed to attach overnight. For an assay using a COL-I gel-coated plate, 50 μL of 0.2% atelocollagen solution was added to each well of a 96-well plate and incubated at 37 ºC for 2 h, before HHUA cells (5,000 cells/150 μL·well) were seeded. After incubation for 0 h, 24 h, and 48 h, 20 μL of Cell Counting Kit-8 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was added to each well, and the plate was incubated at 37 ºC for another 4 h. The absorbance was measured at 450 nm using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Walthman, MA, USA). The cell number was plotted as a percentage relative to that at 0 h. Error bars represent standard deviation (n = 4).

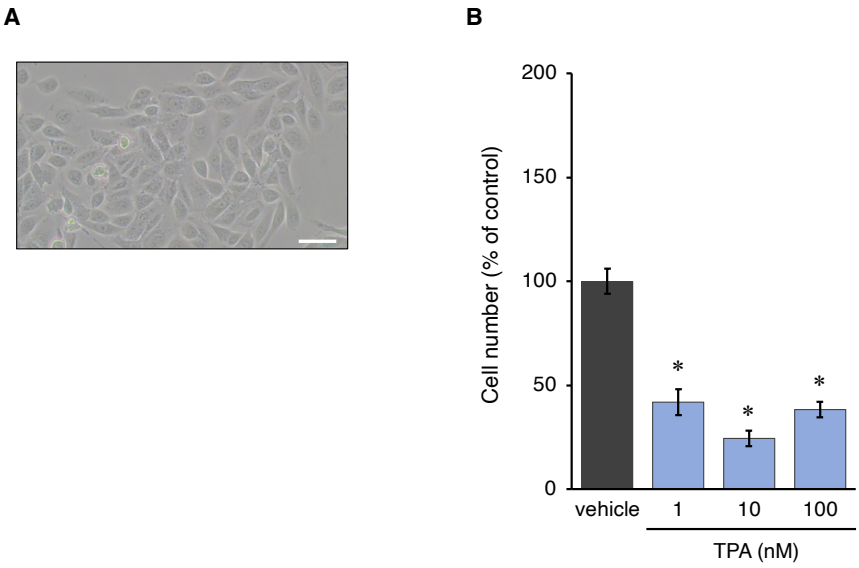

Figure S3. (A) The morphology of HHUA cells cultured on a commercial COL-I coated dish. Scale bar: 50 μm. (B) Effects of TPA on the proliferation of HHUA cells cultured on a commercial COL-I coated plate. Error bars represent standard deviation ($n = 4$). * $p < 0.001$ vs. vehicle group (Dunnett's test). COL-I coated dish was obtained from Matsunami Glass Ind., Ltd (Kishiwada, Japan). COL-I coated 96-well plate was obtained from AGC Techno Glass Co., Ltd (Shizuoka, Japan)